



Vitamin D testing: Comparison and limitations of currently employed immunoassay with a novel liquid chromatography and tandem mass spectrometry (LCMS/MS) technique.

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INTRODUCTION

Vitamin D is fat soluble in nature and exists in two main forms, Vitamin D2 and D3. They differ in chemical structure and the way they are manufactured. It is associated with Diabetes, insulin resistance and cancer.¹ In the body 80 – 85% of 25 OH vitamin D is the D3 form. The most potent is 1 alpha hydroxyl form. It is very low in serum and has a short half-life of 4 hours.²

The methods of Vitamin D analysis include Immunochemical methods which use radioactive, Chemiluminescence and enzyme labels and physical detection methods like HPLC & LC-MS/MS. Immunoassay are treated as common but have certain limitations like, it is not specific, unable to differentiate between D3 and D2 form, has low selectivity, cross reactivity issues

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ABSTRACT... Objective: To compare analytical method for 25 hydroxy vitamin D2 and D3 on LC/MS-MS and with routine vitamin D Immunoassay method. **Study Design:** Cross Sectional study. **Setting:** Department of Chemical Pathology and Endocrinology AFIP, Pakistan. **Period:** March 2019 to March 2020. **Material & Methods:** Samples were extracted and a mass spectrometer coupled to high performance liquid chromatography was adopted for quantitation of 25-hydroxyvitamin D2 and D3 in human samples (serum). After validation it was then applied to 120 serum samples from healthy individuals for method comparison. **Results:** The method was validated in terms of accuracy, precision, linearity on calibration curve, Limit of Detection and Limit of Quantitation. Our study showed a statistically insignificant difference in results among both the methods ($p=0.715$). Limit of detection (LOD) was 2.49 ng/ml and limit of quantitation (LOQ) was 3.9 ng/ml for both the metabolites. Percentage RSD was 0.8% and 1.3% for D2 and D3 respectively. This method has an advantage of minimal cross-reactivity with 24,25 hydroxy vit D and 25,26 di- hydroxy vit D metabolite than the routinely used assays. **Conclusion:** This methodology will be helpful in guiding patient management and assess possibility of malabsorption syndrome in patients on D2 therapy. It can give highly cost effective reliable results of Vitamin D at a tertiary care setting which has an already installed LCMS/MS with huge workload, as compared to costly Immunoassay method.

Key words: 25 Hydroxy vitamin D2, 25 Hydroxy vitamin D3, Immunoassay, LCMS, Validation.

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and interferences.³ LCMS/MS is considered as standard and reliable method for analysis. LCMS/MS requires extraction to avoid matrix effects and interferences which makes it laborious.

Keeping in view the issues encountered by immunoassays, and increasing prevalence of vitamin D deficiency there is a need of a robust, sensitive and precise method for evaluation which can quantify D2 & D3 fractions of 25 OH-vitamin D for correct measurement of deficiency & subsequent management.⁴ Current study was conducted with the aim of method development on LC/MS-MS, its comparison with the routinely used immunoassay. Availability of a purchased instrument in the department made this LC/MS-MS method more economical than routine Immunoassay method and it puts less financial

burden over laboratory because of its low running cost.⁵ Both endogenous and exogenous vit D produced are metabolized in liver. Vitamin D is converted to 25-HydroxyVitamin-D or calcidiol under the catalytic effect of enzyme 25 Hydroxylase in the liver (Bikle, 2017). The vitamin D taken orally produce a fast but less sustained increase in total vitamin D stores as compared to vitamin D that is absorbed from the skin. VDBP has a less affinity for vit D2 as compared to vit D3. Also the half-life of vit D2 is shorter than D3. These facts make therapeutic doses of D2 less potent and effective in increasing the overall vitamin D status unlike Vit D3. Vitamin D is normally stored in the liver. But if larger doses are taken then the extra Vit D is stored in adipose tissues. After saturation of these storing sites the excess vitamin D reaches toxic levels. Vit D is also converted into inactivate metabolites by liver through P450 enzyme system. The concentration of 1, 25- Dihydroxy Vitamin D in blood depends on the availability and the activity of the renal enzymes (1-Alpha hydroxylase & 24-Alpha hydroxylase). These are mainly regulated by the factors like PTH, Serum Calcium levels, Serum Phosphate concentration and fibroblast growth factor 23 (FGF-23). This study conducted with the aim of comparison of vit D analysis by LC/MS and immunoassay.

MATERIAL & METHODS

Total sample size was 120. For method comparison 120 samples from disease free participants were taken and analyzed on both the techniques. Sample size calculated through given protocol of method comparison. The technique of non-probability convenient sampling was incorporated. Patients coming to Armed forces institute of pathology between the ages of 18 to 80 years for vitamin D Analysis were included while pregnant and lactating women, patients with acute and chronic illness (Tuberculosis, CKD, CCF, CLD) and history of alcohol intake were excluded from the study.⁶ 05 ml baseline venous blood sample was taken in clot separator /gel tube at the start of study. After centrifugation of patient's samples. Serum was divided into two aliquots. One of the aliquot was immediately processed for vitamin D by chemiluminescent

assay kit on Advia (Centaur) and second aliquot was stored at temperature -80°C for further analysis of vitamin D by LCMS/MS.⁷ The detailed protocol is described below. The proposal was presented to the AFIP Institute's review board and ethical approval (Number: MP-CHP18-4/READ-IRB/19/648) was taken before initiating the project.

Following calibrators were obtained by Ceriliant®, a Sigma Aldrich company for method development. Serial dilutions were prepared accordingly.

- 25 Hydroxy vitamin D3 in 5 $\mu\text{g}/\text{ml}$ vial
- 25 Hydroxy vitamin D2 in 50 $\mu\text{g}/\text{ml}$ vial
- 25 Hydroxy vitamin D3-d6 $\mu\text{g}/\text{ml}$ vial (was used as the internal standard)

Stock solutions were prepared accordingly & placed at -20°C in amber vials protected from light. They were found to be stable for a period of 3 months under these conditions. By keeping the solutions in dark, light- induced degradation of the analyte was avoided.⁸ Other reagents used includes methanol, N-Hexane, ethylacetate, formic acid, HPLC grade ultrapure water. All solvents were LC.MS grade. Blood sample was collected in serum separator tube. After centrifugation serum was separated. In 500 μl serum 100 μl internal standard was added and vortexed briefly, followed by addition of 400 μl of methanol and vortexed for 1 min and kept for incubation for 10 minutes at the room temperature. Afterwards, 1000 μl of Ethyl acetate: n- hexane (1:1) mixture was dispensed. The tubes were rotated for 10 minutes followed by 15 minutes centrifugation at 15,000 rpm.⁹ The supernatant was collected in a separate tube and kept for drying under nitrogen for 10 minutes. Finally the sample was reconstituted in 200 μl of methanol: water (75:25) and injected for analysis. POROSHELL column (120EC – C18) having dimensions 2.1 x75mm, 2.7 micron was utilized which physically separated the metabolites. Injector volume was kept 20 μl while the chromatographic column was maintained at a temperature of 50°C . Flow-rate was maintained as 0.5mL/min for achieving separation. The LC module parameters and gradients (mobile phase) were adjusted accordingly. An electro

spray ionization (ESI) mode with positive polarity was used. While nitrogen gas was utilized as a desolvation and collision gas.

The data collected was statistically analyzed by using the software SPSS 22 i.e Statistical package for Social Sciences. Qualitative variables computed as frequency and percentages while quantitative variables computed as median IQR and mean and standard deviation. Kolmogorov-Smirnov test applied to check data distribution. In inferential statistics Wilcoxon test and Mann-Whitney U test applied and significance level less than p value 0.05 is considered. The conversion factor for 25-OHD₂ and 25-OHD₃ is 2.496.1.¹¹

RESULTS

A total of 120 healthy subjects were recruited in the current study. Median age was 43 in healthy subjects. There were 40(33.3%) males and 80(66.7%) females in the study. 83(69.2%) subjects were from urban areas while the remaining 37(30.8%) were rural. For these 120 healthy individuals, whose vit D levels were

analyzed on immunoassay, 40(33.3%) were in category of vitamin D deficiency, 45(37.5%) falls in the category of vitamin D insufficiency and 33(27.5%) were having sufficient vitamin D levels while only 2(1.7%) were having vitamin D toxicity. Gender wise distribution of vitamin category is shown in (Figure-1) Characteristics of immunoassay and LC/MS has been described in Table-I. Comparison of both the methods used for analysis of 25 OH D shown in scattered plot in Figure-2.

Median of 25OH vitamin D (D₂ + D₃) estimated by LCMS/MS and immunoassay technology were compared by non-parametric inferential statistics. The p value of less than 0.05 was regarded as significant.¹⁴ Median of 25 OH Vitamin D by immuno assay was 58.50 while median of total 25 OH Vitamin D analyzed by LCMS was 42. In our study Mann Whitney U test revealed p value of > 0.05, which proved that the difference between the two vitamin D methods i.e immunoassay and LCMS/MS technology is insignificant. Both of these methods can be used interchangeably.

Parameters	Advia Centaur(Immunoassay)	LC/MS
System Description	Random access immunoassay system	Liquid chromatography/Mass spectrometry
Method	Chemiluminescence	Liquid chromatography
Chemiluminescence agent	Acridinium ester	5 % methanol in 0.1% formic acid(Mobile phase)
Throughput	180 test /Hour	Manual (operator dependent)
Type of sample	Serum, plasma - assay dependent	Serum, plasma
Mode	Calibration curve	Calibration curve
Concentration Units	RLU	Mass/ charge ratio
Run time	15 min	7 Min
Sample Volume	20 µL	10 µL
Assay Range	10.5 nmol/L to 375 nmol/L (4.2 to 150 ng/ml).	3.9 - 200 ng /ml
Assay Accuracy	99%	99.8%
LOD/LOQ	4.2 ng/ml	2.49/3.9 ng/ml

Table-I. Characteristics of Immunoassay and LC/MS.

Variables	Mean±SD	Median	P-Value
Immunoassay	77.30±55.029	58.50	0.551
LC/MS	55.45±47.49	42.0	0.553
Male	75.28±44.6	55.50	0.926
Female	65.11±35.18	53.50	0.833

Table-II. Descriptive statistics of variables.

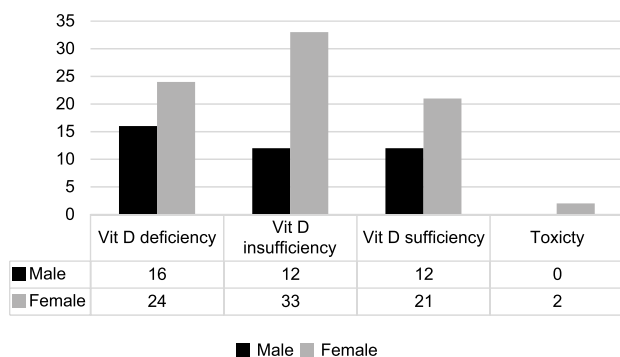


Figure-1. Gender wise distribution of Vitamin D categories on Immunoassay.

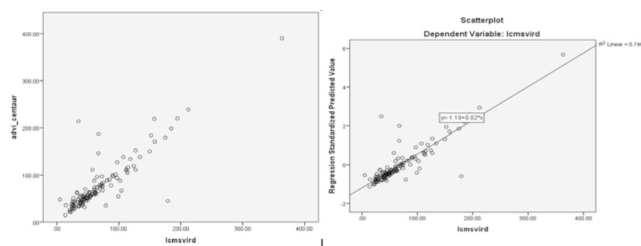


Figure-2. Comparison of both the methods used for analysis of 25 OH D.

DISCUSSION

The best indicator of vitamin D status is 25 OH vitamin D and its determination is no doubt an important area in analytical biochemistry.¹⁵ This study proves that the vitamin D deficiency is more common in Pakistani women as compared to men. A similar study was reported in southern-china which reports that the 25 hydroxy vitamin-D levels in the males were higher as compared to females¹⁶, & the prevalence of vitamin D deficiency was higher in women as compared to men.¹⁷ Use of deuterated analogs as internal standards improves their detection. Some companies are also offering online an automated sample pretreatment kit which increases the throughput.¹⁸ These techniques decrease the man power and human manipulation thereby decreasing operator errors and chemical hazards by use of reagents. One added advantage of LCMS/MS is high sensitivity and specificity.¹⁹

In this study the methanol precipitation is used for the extraction of samples followed by a liquid-liquid extraction step by n-hexane: ethyl acetate (v/v). This technique produces a comparatively pure extract. It was observed in current study

that the vitamin D2 was estimated in only 16% of the healthy individuals. This may be due to lack of vitamin D2 supplementation and inadequate diet. A similar study conducted by Wang et. al reported that out of 120 healthy population, only 21% of the samples were having detectable D2 concentration.²⁰ Mean by immunoassay and LCMS is significantly not different as shown. This study gives a view that LC/MS technique is more sensitive than immunoassay but it is laborious method. This study proves that there is no statistically significant difference between means of both methods, so it can be used interchangeably.

CONCLUSION

The described method utilizing LCMS technology allows detection and quantitation of 25 OH D2 and D3, separately in human blood which will be helpful in guiding the management of patients. This LC-MS/ MS method is highly sensitive, specific and has less cross reactivity with other vitamin D metabolites. It can give highly cost effective reliable results of Vitamin D at a tertiary care setting with mega workload which has already installed equipment as compared to costly Immunoassay method.






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2	Muhammad Aamir	Conception of idea, Drafting, Manuscript proof reading.	
3	Zujaja Hina Haroon	Design and implementation of research project, feasibility, Drafting.	
4	Sobia Irum	Constant supervision throughout the completion of research project, Manuscript proofreading.	
5	Qurat ul Ain	Manuscript proofreading, Statistical contribution drafting.	
6	Nisar Ahmed	Sample analysis, Instrument handling, results rechecking.	